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Short Communication

Diversity of the candidate phylum *Poribacteria* in the marine sponge *Aplysina fulva*

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Abstract

Poribacterial clone libraries constructed for *Aplysina fulva* sponge specimens were analysed with respect to diversity and phylogeny. Results imply the coexistence of several, prevalently "intraspecific" poribacterial genotypes in a single sponge host, and suggest quantitative analysis as a desirable approach in studies of the diversity and distribution of poribacterial cohorts in marine sponges.

Key words: marine sponges, bacterial diversity, bacterial phylogeny, 16S rRNA gene.

The study of microbial communities based on 16S ribosomal RNA (rRNA) gene analysis has unveiled more than 25 recognized bacterial phyla associated with marine sponges (Hentschel *et al.*, 2006; Taylor *et al.*, 2007; Webster *et al.*, 2011). The recently proposed candidate phylum *Poribacteria* (Fieseler *et al.*, 2004) appears to be strictly associated with, or at least enriched in, marine sponges (Fieseler *et al.*, 2004; Hentschel *et al.*, 2006; Lafi *et al.*, 2009; Webster *et al.*, 2010). The analysis of the first *Poribacteria* genome fragment revealed a particular genomic similarity between this candidate phylum and *Planctomycetes*, *i.e.* the presence of a 16S rRNA locus that is physically separated from the other rRNA genes (*i.e.*, an unlinked *rrn* operon) (Fieseler *et al.*, 2006). These two phyla share the peculiar feature of intracellular compartmentalization and consequent presence of a membrane-bound nucleoid in their cells (Fieseler *et al.*, 2004; Fuerst, 2005). However, only two of 27 poribacterial open reading frames shared significant homology with those of the planctomycete *Rhodopirellula baltica* (Fieseler *et al.*, 2006). These observations suggest that the two phyla may have shared a common evolutionary ancestor to the exclusion of other bacteria (Fuerst *et al.*, 2005; Fieseler *et al.*, 2006; Wagner and Horn, 2006), but have since become divergent at the primary sequence level. Recent advances made by single cell genomics revealed that the so far uncultured *Poribacteria* are likely mixotrophic organisms with a Gram-negative cell wall, possessing at least two polyketide biosynthesis gene clusters and several putative symbiosis factors such as adhesins and adhesin-related genes (Siegl *et al.*, 2011). As a recent candidate phylum with no cultured representatives, still little is known about the diversity and distribution of the *Poribacteria* worldwide. In the present study, we generated 97 poribacterial 16S rRNA gene sequences retrieved from *Aplysina fulva* (Verongida, Demospongiae) on the Southeast Brazilian coast. We provide a quantitative assessment of their diversity, and report on their phylogenetic affiliation within the phylum. To the best of our knowledge, this is the first registration of the *Poribacteria* in the South Atlantic.

Aplysina fulva Pallas 1766 specimens were sampled at Caboclo Island (22°45'18.81" S, 41°53'23" W) and Tartaruga Beach (22°45'20.67" S, 41°54'13.53" W) in Búzios, Rio de Janeiro, Brazil. Sponge sampling (triplicate

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specimens per site) and total community DNA (TC-DNA) extraction with the Fast DNA Spin Kit for Soil (MP Biomedicals, LLC, Illkirch, France) were performed as explained by Hardoim *et al.* (2009).

Amplification of *Poribacteria*-specific 16S rRNA gene fragments from sponge TC-DNA was carried out using the primer pair POR389f and POR1130r and procedures of Fieseler *et al.* (2004). Amplicons from the same sampling site were pooled and purified, and one clone library was generated for each site - *i.e.* Caboclo Island (PC) and Tartaruga beach (PT) - with the pGEM®-T easy vector cloning kit following the supplier's recommendations (Promega, Madison, WI, USA). Cloned fragments with right size were purified and sequenced with the chain termination method, as described by Hardoim *et al.* (2009), with the primer POR389f. Assessments of sequence quality and chimera checks were performed, after which 49 and 48 sequences from PC and PT, respectively, were selected for further analyses. Sequences were deposited in the EMBL database under the accession numbers FN356776 to FN356876.

Evolutionary distances calculated with the Kimura 2-parameter were applied to generate pairwise similarity matrices with the DNADIST software (http://cmgm.stanford.edu/phylip/dnadist.html). These were used as templates for the assignment of sequences to operational taxonomic units (OTUs) using the furthest-neighbour method as implemented in the DOTUR software (Schloss and Handelsman, 2005). The frequency data assigned to 'unique' OTUs – defined at 99, 97 and 95% levels of similarity - were employed for the construction of rarefaction curves, estimation of theoretical richness using the Chao1 (Chao 1984; Chao and Lee 1992; modified by Colwell, R.K. – http://viceroy.eeb.uconn.edu/estimates) and ACE (Chao and Lee 1992; Chao *et al.*, 1993) estimators, and calculation of Shannon's diversity indices. In addition, to determine whether the two poribacterial libraries were significantly different in their composition, library shuffling analysis using the program mothur was performed (Shloss *et al.*, 2009).

For phylogenetic inference, all sequences were aligned using the SINA web aligner (Pruesse *et al.*, 2007) and imported into the SILVA 16S rRNA database version 102 using the parsimony tool as implemented in the ARB software (Ludwig *et al.*, 2004). Alignments were manually refined using the ARB alignment tool. The 16S rRNA gene sequences of type strains and uncultured representatives of *Poribacteria*-related phyla, such as *Planctomycetes*, *Verrucomicrobia*, *Chlamydia*, *Lentisphaerae* and the candidate phylum OP3 (Fieseler *et al.*, 2004; Wagner and Horn, 2006), were included in the alignment procedure. Poorly-aligned sites (where the assumption of homology could not be made with confidence) were identified and excluded from further analysis using PAUP* (ver. 4.0b10; Swofford 2003). An appropriate evolutionary model was then determined using MrModeltest (ver. 2.3; Nylander 2008) – and this was the general-time reversible model (GTR, Rodriguez *et al.*, 1990) with a discrete gamma-distribution of among-site rate variation (Γ_4) and a proportion of invariant sites (I). An optimal maximum likelihood tree was determined using RAxML (ver. 7.0.4-MPI; Stamatakis 2006) with 100 replicates, each starting from a random tree, with the GTR+Γ_4+I model. Maximum likelihood bootstrap support was determined with the same software and model using 300 replicates with thorough final optimisation of the tree from each replicate (option "-f i"). A Bayesian MCMC analysis was conducted using MrBayes (ver. 3.2.1; Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) each of 2 runs using 4 chains (Metropolis-coupling) for 2 million generations, sampling every 1000 generations, and using the GTR+Γ_4+I model. All other options, including priors, were default values. The 'burn-in' period before the MCMC reached stationarity was determined by plotting the likelihood through time using the plotting programme GNUPlot (Williams *et al.*, 2004); stationarity was assumed to have been reached when the curve plateaued. Tree sets from the posterior distribution of the two independent runs were concatenated to form the sample of trees assumed to be randomly sampled from the posterior probability distribution, and 50% majority-rule consensus trees were constructed in P4 (Foster 2004). The marginal likelihood of the MCMC analysis was estimated using the equation 16 of Newton *et al.* (1994) as implemented in P4.

All sequences retrieved in this study resembled poribacterial 16S rRNA gene sequences previously deposited in relevant databases. Rarefaction analysis revealed that, at 99 and 97% sequence similarity cut-offs, both the observed and estimated richnesses of the PC library were lower than those registered for the PT library (Table 1). No difference

Table 1 - Richness estimates (Chao1 and ACE, average values) and Shannon diversity measures for poribacterial clone libraries at 99, 97 and 95% similarity cut-off levels.

Libraries	N[a]	OTUs[b]	Richness		Diversity
			Chao1	ACE	Shannon
99%PC[c]	49	10	12	14.06	1.49
99%PT[d]	48	19	28	38.36	2.54
99%PC+PT	97	25	36	39.82	2.38
97%PC	49	4	4	4.54	0.70
97%PT	48	7	7.5	9.24	1.48
97%PC+PT	97	9	9	9.57	1.57
95%PC	48	2	2	2.00	0.33
95%PT	49	2	2	2.00	0.29
95%PC+PT	97	2	2	2.00	0.33

[a]Number of analysed sequences. [b]Number of observed Operational Taxonomic Units. [c]PC - Caboclo Island clone library. [d]PT – Tartaruga beach clone library.

in richness between the libraries was observed at a 95% sequence similarity cut-off, whereby only two OTUs per library could be determined (Table 1). Library shuffling analysis revealed that, at 99% and 97% evolutionary distances, PT significantly covered the diversity found in PC ($p = 0.0008$), whereas the opposite was observed for PC in comparison with PT ($p > 0.05$). Indeed, of the 25 OTUs determined at 99% similarity cut-off for both libraries, 14 (OTUs 12 to 25) contained only sequences from the PT library, embracing 23 sequences in total. Conversely, 29 sequences from the PC library were assigned to a single OTU (OTU 2, representing the most frequent poribacterial sequence retrieved in this study). As expected, for PC+PT the observed richness at 99 and 97% was higher than that determined for each library separately. Richness estimates obtained for the PT library at both cut-off levels approached the values calculated for PC+PT, especially when the ACE estimator was used (Table 1). The same occurred for the calculated Shannon diversity indices (Table 1), reflecting the higher poribacterial 16S rRNA gene variability found in the PT library. Rarefaction curves generated at 99% similarity cut-off did not reach a plateau for PT and PC+PT libraries (data not shown), suggesting that, at this level, libraries of larger sizes would be required to successfully cover poribacterial 16S rRNA gene richness in a single sponge species. Coverage of poribacterial richness increased substantially in PC, PT and PC+PT libraries when analyses were performed with uniqueness threshold set at 97% sequence similarity. At this level, rarefaction curves revealed satisfactory sampling of poribacterial richness, with both Chao1 and ACE richness estimates reflecting very well the observed richness in each library (Table 1).

Phylogenetic analysis encompassed 157 "taxa": 77 non-redundant poribacterial sequences - 74 from sponges and 3 from seawater - and 80 sequences from the "PVC superphylum". The data included 1103 sites, after removing ambiguously aligned sites, of which 594 were parsimony informative. In Figure 1 the maximum likelihood tree (-ln likelihood: 22887.766493) is presented, with bootstrap percentages indicated at nodes. The tree supports the monophyletic status of the candidate phylum *Poribacteria* as observed elsewhere (Fieseler *et al.*, 2004; Lafi *et al.*, 2009; Siegl *et al.*, 2011; Simister *et al.*, 2012). Accordingly, our analysis also supports the existence of poribacterial clades III and IV, determined by Lafi *et al.* (2009). Sequences previously assigned by these authors as members of poribacterial clades I and II belonged to a large and diversified cluster clearly distinct from groups III and IV, with bootstrap support (Fig. 1). Hereafter, we will refer to clades I and II of Lafi *et al.* (2009) as "clade I-II". Overall, tree topology inferred by maximum likelihood was well supported by Bayesian phylogenetic inference (Figure 1). All poribacterial sequences recovered from Caboclo Island and Tartaruga Beach fell into clade I-II. Twenty-five of these sequences are shown in Figure 1, and represent all OTUs determined by rarefaction analysis at 99% similarity cut-off. "Site" or "host-specific" poribacterial subgroups with phylogenetic support could be observed (Fig. 1). They were composed of highly similar sequences retrieved *e.g.* from *Agelas* sponges (clones AD_Pori 13, ACAP1 and AD_Pori15), *Aplysina fulva* (clones PT30, PT34 and PT49) and from the Great Barrier Reef (clones PCPO8, RGPO96, RGPO105). Also to note in Figure 1 is the positioning of two poribacterial sequences retrieved from seawater (clones HF770_17N21 and HF200_24O18; Pham *et al.*, 2008), which did not affiliate with any of the groups I-II through IV. However, clone SWB-Pla-31, also obtained from seawater (Mohamed *et al.*, 2010), fell into cluster IV (Fig. 1), which is dominated by sponge-derived sequences. Interestingly, whereas the former sequences derive from ocean water samples, the latter was retrieved from seawater collected near sponge species.

In 2004, the candidate phylum *Poribacteria* was proposed on the basis of < 75% 16S rRNA gene sequence similarity with all other known bacterial phyla, and of its association with different marine sponge species. The present study constitutes the first registration of the *Poribacteria* in the South Atlantic. It extends the known geographical range of occurrence of *Poribacteria* and supports the contention that they have a cosmopolitan distribution at a global scale (Lafi *et al.*, 2009).

Overall, phylogenetic inference strengthens previous observations on the absence of clear relationships between geographic location, sponge host and poribacterial phylogenies (Lafi *et al.*, 2009). The detection of specific poribacterial signatures, either related with a given sponge host or location, might very much depend on the level of genetic resolution applied in the analyses, with stringent levels favouring the observation of "specific" patterns, as exemplified by some poribacterial subgroups with phylogenetic support that could be detected in this study (Figure 1). It is, however, not possible to rule out the effects of low sampling sizes on such observations, and therefore conclusions on possible *Poribacteria* - sponge host - location specificities might be premature. Conversely, recently deposited poribacterial sequences from *Ircinia strobilina* (Mohamed *et al.*, 2010) displayed substantial diversity as they were affiliated with clades I-II, III and IV (clones "IS-Pla" in Figure 1). This exemplifies the presence of varied poribacterial genotypes in one single host species, as previously observed for *Aplysina aerophoba* and *Rhabdastrella globostellata* (Lafi *et al.*, 2009) and quantitatively assessed for *Aplysina fulva* in this study.

Recently, next generation sequencing (*i.e.* 454 pyrosequencing) revealed a sharp increase in the relative abundance of poribacterial 16S rRNA gene tags in sponge *vs.* seawater samples collected at the hosts' proximities (Webster *et al.*, 2010). In as far as sponges did not affect the thus surveyed seawater samples, since these have been sampled at the time of sponge spawning, this outcome suggests envi-

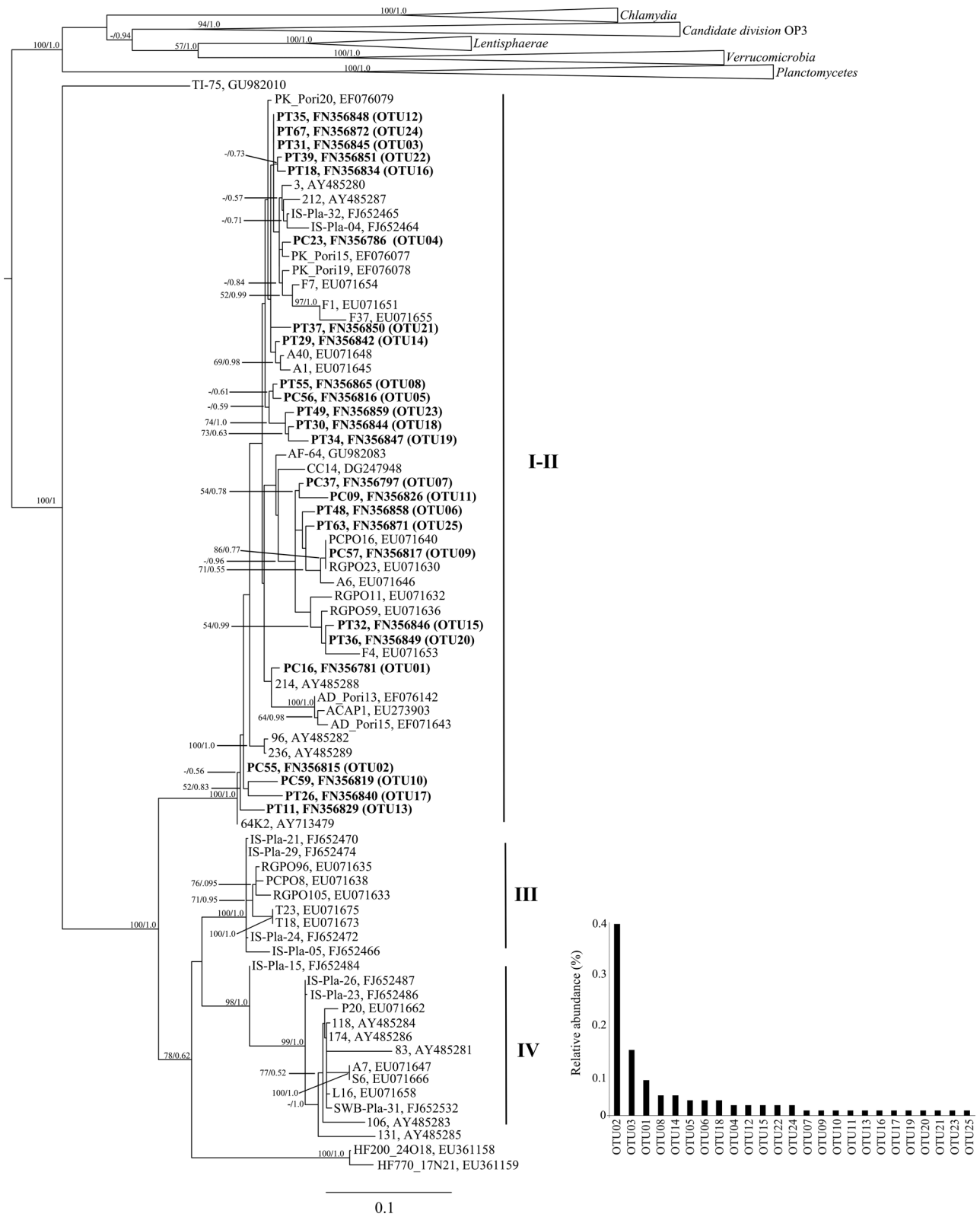

Figure 1 - Phylogenetic inference of poribacterial 16S rRNA sequences. The Maximum Likelihood tree is shown, with sequences retrieved in this study highlighted in bold. Numbers at tree nodes are bootstrap values and posterior probabilities calculated in Maximum Likelihood and MCMC Bayesian analyses, respectively, and values above 50 / 0.5 are shown. The graphic at the right bottom is an abundance rank of 25 *Aplysina fulva*-derived poribacterial OTUs determined at a 99% similarity cut-off threshold. PC: sequences from the Caboclo Island clone library. PT: sequences from the Tartaruga Beach clone library. I-II, III and IV: poribacterial phylogenetic groups delineated in this study, following the nomenclature of Lafi *et al.* (2009).

ronmental acquisition and accumulation of poribacterial cells by sponge hosts from seawater. Such a mode of transmission could well explain the observation of genetically similar poribacteria inhabiting diverse sponge hosts over wide geographical scales. However, it has been demonstrated that vertical transmission of poribacterial symbionts in sponges is also likely to occur. For instance, a poribacterial 16S rRNA gene sequence was reported in a *Corticium* sp. embryo (Sharp *et al.*, 2007). Furthermore, Schmitt *et al.* (2008) detected poribacterial 16S rRNA gene sequences in both adult and embryo specimens of *Agelas conifera*, thus showing that these bacteria may effectively be vertically transmitted in sponges (Schmitt *et al.*, 2008). Thus, the growing body of evidence points to the recognition of the *Poribacteria* as marine sponge symbionts that might exhibit both vertical and environmental modes of transmission in the association with their hosts. Poribacterial 16S rRNA genes have mostly been studied in a more qualitative approach, whereby sequence representatives are solely used in the inference of phylogenetic relationships. Although such an approach is efficient in demonstrating that similar poribacterial sequences occur across large geographic scales and in different sponge hosts, it does not enable assessments of diversity and structure within the group in a particular sample, or variations thereof across multiple samples. Here, the examination of the PC+PT clone library using 99% gene similarity cut-off resulted in a total of 25 observed poribacterial OTUs, delivering an estimated "OTU richness" of 36 and about 40 as determined by Chao1 and ACE richness estimators, respectively (Table 1). Such numbers drop to 9 observed and about 9-10 theoretical poribacterial OTUs in this library when the cut-off criterion used is 97%. We further observed that OTUs determined at both confidence thresholds displayed frequency abundance ranks typical of natural biological assemblages (see Fig. 1 for abundance ranks of OTUs at 99% cut-off). Taken together, these results imply the coexistence of several poribacterial genotypes in a single sponge host, and are indicative of a prevalently "intra-specific" poribacterial genotypic diversity in *A. fulva* on the basis of 16S rRNA gene diversity assessments. Interestingly, a recent next generation 454 pyrosequencing study ranked the *Poribacteria* as the third most diverse bacterial phylum retrieved from 32 marine sponges collected worldwide and, in accordance with this survey, several poribacterial OTUs at 97% similarity cut-off could be retrieved from a single host species (Schmitt *et al.*, 2012).

Dedicated, quantitative approaches to poribacterial diversity and composition are required to and hold promise in increasing our understanding of the degrees of endemism and cosmopolitanism of these symbionts in marine sponges. It is thus far not known to what extent the poribacterial 16S rRNA gene diversity observed in a sponge host, such as addressed in this study, translates into diversified functional attributes of the corresponding symbionts. Future research aimed at unveiling the functional and genomic features of the *Poribacteria* will substantially improve current knowledge of their potential roles in marine sponges. In this context, addressing niche partitioning among genotypically distinct *Poribacteria* in marine sponges might constitute a rewarding challenge to be pursued.

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Supplementary Material

Table S1. Description of poribacterial sequences used in phylogenetic analysis (Figure 1).